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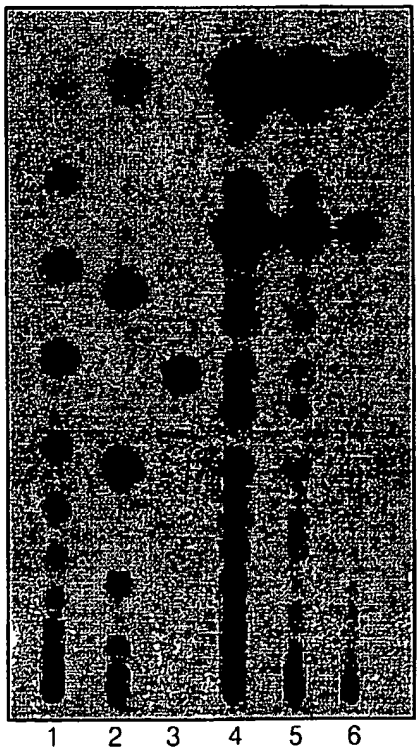
(54) Title: METHOD FOR THE PRODUCTION OF THERMOSTABLE AND ACID-STABLE OLIGOSACCHARIDE BY USING DEXTRANSUCRASE



(57) Abstract: The present invention relates to the methods for manufacturing thermostable and acid-stable oligosaccharides from high concentration of sucrose comprising the steps of adding dextransucrase derived from Leuconostoc mesenteroides sp. in 0.5-5M sucrose solution, and recovering the produced oligosaccharides. The oligosaccharides manufactured according to the present invention can be used as the sweeteners for the food such as beverages requiring thermostable and acid-stable properties.

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METHOD FOR THE PRODUCTION OF THERMOSTABLE AND ACID-STABLE OLIGOSACCHARIDE BY USING DEXTRANSUCRASE

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a method for manufacturing thermostable and acid-stable oligosaccharides.

2. Description of the Prior Art

Recently, the excess uptake of sucrose and other existing saccharides causes many problems such as decayed tooth, obesity, diabetes and other adult diseases. In order to overcome these problems, oligosaccharides as the new kind of alternative saccharides, which have become materials of natural food, have been developed via biotechnology.

In general, oligosaccharides have the degree of polymerization (DP) of 2 ~ 10 monosaccharides (molecular weight of 300 ~ 2,000) that are dehydrated/condensed by combination of glycosidic linkages regardless of the kind of component saccharides. Oligosaccharides, which are commercially produced now, are fructo-oligosaccharides, isomalto-oligosaccharides, malto-oligosaccharides and galacto-oligosaccharides. Researches about soybean-oligosaccharides and xylo-oligosaccharides are in process.

Fructo-oligosaccharides are saccharides having DP of 3~5, and made by combining 1~3 fructose molecules with sucrose using β-fructofuranosidase (FFase) that is fructose transfer enzyme of sucrose. The fructo-oligosaccharides are finally produced after making the product by enzyme react on sucrose solution and performing the processes of decoloration, filteration and demineralization of it.

Isomalto-oligosaccharides are saccharides wherein

glucose molecules have the combination of α-1, 6 linkage. After starch solution as substrate is dextrinized using α-amylase, β-amylase and transglucosidase, then, are doing saccharification and transition of it at the same time.

Malto-oligosaccharides are saccharides wherein glucose molecules have the combination of α-1, 4 linkage. Its major component is maltoriose or maltotetraose(more than 50% in the whole quantity of saccharides). Although the process of synthesizing malto-oligosaccharides is similar to that of isomalto-oligosaccharides, it is different that α-amylase, β-amylase and plunanase are used in the saccharification and transition.

Galacto-oligosaccharides are saccharides consisting of galactose and glucose. After enzymes for transferring saccharides (β-galactosidase) are made to react on lactose used as substrate, galactose is added thereto. Then, galacto-oligosaccharides are synthesized.

The commercially produced malto-oligosaccharides such as oligosaccharides consisting of glucose are resistant to acid and heat treatment. Yet they are not sweet. On the contrary, the fructo-oligosaccharides are sweet while they are not resistant to acid and heat treatment. As a result, both of them are restricted in the use as additives or sweeteners in food that needs heat treatment during the process.

Dextransucrase (EC 2.4.1.5) are enzymes for synthesizing glucan from sucrose. They are produced from microbes of *Leuconostoc* and *Streptococcus* sp. mainly. The mechanism of dextransucrase reacting on sucrose is as follows.

n sucrose → (glucose)n-m-w + n-m fructose +
m leucrose + w glucose

The main products of these enzyme reaction are glucan

having molecular weight about 10^7~10^8 Da and fructose. Glucose and leucrose (5-O-α-D-glucopyranosyl-D-fructopyranose) are produced as the side products.

The Korean Patent Application No. 1998-24355 discloses a method for producing innovative oligosaccharides by using dextransucrase derived from mutant of Leuconostoc mesenteroides. In this method, substrate is sucrose, and the acceptor is maltose, gentiobiose, raffinose or lactose. However, the entire quantity of carbohydrate is fixed at low concentration of 100 mM in this method. There is not provided a method for producing oligosaccharides at high concentration of sucrose (500 mM-4M). On the contrary, a method of the present invention provides a method for producing oligosaccharides by using only sucrose without any acceptor. The oligosaccharides produced in this way are thermostable and acid-stable as well as sweet unlike ones fabricated in the Patent Application No. 24355.

SUMMARY OF THE INVENTION

An object of the present invention is to provide thermostable and acid-stable oligosaccharides.

Another object of the present invention is to provide a method for manufacturing thermostable and acid-stable oligosaccharides.

To achieve the above-described objects, the present invention provides a method for manufacturing thermostable and acid-stable oligosaccharides.

The present invention also provides oligosaccharides manufactured by the above-described method.

The method for manufacturing thermostable and acid-stable oligosaccharide comprises the steps of cultivating Leuconostoc mesenteroides sp. that produce dextransucrose in

0.5-5M sucrose solution and recovering the produced oligosaccharides.

The method for manufacturing thermostable and acid-stable oligosaccharides comprises the steps of adding dextransucrase derived from Leuconostoc mesenteroides sp. in 0.5-5M sucrose solution and recovering the produced oligosaccharides.

Dextransucrase of the present invention is an enzyme for synthesizing dextran derived from Leuconostoc mesenteroides. For example, it includes strains such as Leuconostoc mesenteroides NRRL B-742(ATCC 13146), Leuconostoc mesenteroides NRRL B-1299(ATCC 11499), Leuconostoc mesenteroides NRRL B-512(ATCC 10830) or mutants thereof. It also includes dextransucrase derived from E.coli DH5a/pFMCM(KCTC 0859BP) entrusted by the present inventor or transformants obtained by the introduction of dextransucrase genes separated from the mutatants.

In the method of the present invention, maltose is further added to sucrose, thereby resulting in the stimulation of thermostable and acid-stable oligosaccharides synthesis.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be explained in terms of exemplary embodiments described in detail with reference to the accompanying drawings, which are given only by way of illustration and thus are not limitative of the present invention, wherein:

Figure 1 is a TLC result illustrating the pattern of the biosynthesized product using dextransucrases prepared from E.coli DH5α/pFMCM(KCTC 0859BP) L. mesenteroides B-742(ATCC 13146) in sucrose solution having high concentration.

Lane 1, maltodextrine standard material mixture;

Lane 2, iso-maltodextrine standard material mixture;

Lane 3, panose;

Lane 4, 4M sucrose+E.coli DH5α/pFMCM dextransucrase;

Lane 5, 2M sucrose+E.coli DH5α/pFMCM dextransucrase;

Lane 6, 2M sucrose + L. mesenteroides NRRL 742 dextransucrase.

Figure 2 is a TLC result illustrating (1) the degree of sucrose use by E.coli DH5α/pFMCM dextransucrase when maltose was added and (2) the size of the biosynthesized dextran.

Lane 1, maltodextrin standard material mixture;

Lane 2, isomaltodextrin standard material mixture;

Lane 3, sucrose;

Lane 4, maltose;

Lane 5, 4M sucrose + dextransucrase;

Lane 6, 3M sucrose + dextransucrase;

Lane 7, 2M sucrose + dextransucrase;

Lane 8, 1M sucrose + dextransucrase;

Lane 9, 0.5M sucrose + dextransucrase;

Lane 10, 4M sucrose + 0.4M maltose + dextransucrase;

Lane 11, 3M sucrose + 0.3M maltose + dextransucrase;

Lane 12, 2M sucrose + 0.2M maltose + dextransucrase;

Lane 13, 1M sucrose + 0.1M maltose + dextransucrase;

Lane 14, 0.5M sucrose +0.05M maltose +dextransucrase;

Lane 15, 4M sucrose +0.04M maltose +dextransucrase;

Lane 16, 3M sucrose +0.03M maltose +dextransucrase;

Lane 17, 2M sucrose +0.02M maltose +dextransucrase;

Lane 18, 1M sucrose +0.01M maltose +dextransucrase;

Lane 19, 0.5M sucrose+0.005M maltose+dextransucrase.

Figure 3 is a TLC result illustrating (1) the degree of sucrose use by L. mesenteroides B-742 dextransucrase when maltose was added and (2) the size of the biosynthesized

dextran.

Lane 1, maltodextrin standard material mixture;

Lane 2, isomaltodextrin standard material mixture;

Lane 3, sucrose;

Lane 4, 2M sucrose + dextransucrase;

Lane 5, 2M sucrose + 0.2M maltose + dextransucrase;

Lane 6, 1M sucrose + dextransucrase;

Lane 7, 1M sucrose +0.1M maltose + dextransucrase;

Lane 8, 0.5M sucrose + dextransucrase;

Lane 9, 0.5M sucrose + 0.05M maltose + dextransucrase.

Figure 4 is a TLC result illustrating (1) the degree of sucrose use by L. mesenteroides B-1299(ATCC 11449) dextransucrase when maltose was added and (2) the size of the biosynthesized dextran.

Lane 1, matodextrin standard material mixture;

Lane 2, isomaltodextrin standard material mixture;

Lane 3, panose;

Lane 4, 0.5M sucrose + 0.05M maltose +dextransucrase;

Lane 5, 0.5M sucrose + 0.1M maltose +dextransucrase;

Lane 6, 0.5M sucrose + 0.5M maltose +dextransucrase;

Lane 7, 0.5M sucrose + 1M maltose +dextransucrase;

Lane 8, 0.5M sucrose + 1.5M maltose +dextransucrase;

Lane 9, maltodextrin standard material mixture;

Lane 10, isomaltodextrin standard material mixture;

Lane 11, 3.09M sucrose + dextransucrase;

Lane 12, 2.06M sucrose + dextransucrase;

Lane 13, 1.55M sucrose + dextransucrase;

Lane 14, 1.03M sucrose + dextransucrase;

Lane 15, 0.52M sucrose + dextransucrase;

Lane 16, 0.1M sucrose + dextransucrase.

Figure 5 is a TLC result illustrating the acid-stable oligosaccharides formed by E.coli DH5α/pFMCM dextransucrase at various temperatures.

Lane 1, isomaltodextrin standard material mixture;

Lane 2, oligosaccharides (4M sucrose + E.coli DH5α/pFMCM dextransucrase);

Lane 3-7, pH 2 in 40, 60, 80, 100, 120°C;

Lane 8-12, pH 3 in 40, 60, 80, 100, 120°C;

Lane 13-17, pH 4 in 40, 60, 80, 100, 120°C;

Lane 18-22, pH 5, in 40, 60, 80, 100, 120°C;

Lane 23, maltodextrin standard material mixture.

Figure 6 is a TLC result illustrating the thermostable oligosaccharides synthesized by E.coli DH5α/pFMCM dextransucrase.

Lane 1, maltodextrin standard material mixture;

Lane 2, isomaltodextrin standard material mixture;

Lane 3, panose;

Lane 4, oligosaccharides (4M sucrose + E.coli DH5α/pFMCM dextransucrase);

Lane 5-6, 120°C in 30, 60 min.;

Lane 7-8, 140°C in 30, 60 min.;

Lane 9-10, 160°C in 30, 60 min.

Figure 7 is a picture illustrating the separation result of highly branched oligosaccharides using two-dimensional thin layer chromatography, wherein the reference numbers are designated as follows: lane 17: maltodextrin series; lane 18: highly branched oligo-saccharides; lane 19: the starting point of highly branched oligosaccharides in two-dimensional thin layer chromatography; lane 20: isomaltodextrin series; lane 21: highly branched oligosaccharides using developing solvent of 2:5:1.5 (v/v/v) nitromethane/1-propanol/water (the first development)→85:20:50:50(v/v/v/v)acetonitrile/ethylacetate/1-propanol/water(the second development); and 1-16: each

component material in highly branched oligosaccharides.

Figure 8 is a picture illustrating the analysis result using the thin layer chromatography of degradation product after the enzyme treatment of fractions separated from highly branched oligosaccharides, wherein the reference numbers are designated as follows: lane 1: maltodextrin series; lande 2, 19: highly branched oligosaccharides; lane 20: isomaltodextrin series; (1) lane 3-10: enzyme reaction product of fraction 2 separated from highly branched oligosaccharides; lane 11-18: enzyme reaction product of fraction 3 separated from highly branched oligosaccharides; (2) lane 3-10: enzyme reaction product of fraction 8 separated from highly branched oligosaccharides; and lane 11-18: enzyme reaction product of fraction 9 separated from highly branched oligo-saccharides. Each fraction was treated with each enzyme such as α-, β- and iso-amylase, α- and β-glucosidase, dextranase, α-amyloglucosidase, and invertase.

Figure 9 is a picture illustrating the analysis result using the thin layer chromatography after each fraction separated from highly branched oligosaccharides was treated with dextranase, wherein the reference numerals are designated as follows: lane 1: isomaltodextrin series; lane 2: maltodextrin series; lane 3: highly branched oligosaccharides; lane 4: dextranase degradation product of fraction separated from highly branched oligosaccharides; lane 5: degradation product after isomaltulose was treated with dextranase; lane 6: degradation product after leucrose was treated with dextranase; lane 7: highly branched oligosaccharides; and lane 8: isomalto-oligosaccharides.

Figure 10 is a picture illustrating the analysis result using the thin layer chromatography of the acceptor reaction product using dextransucrase and fractions separated from highly branched oligosaccharides, wherein the reference

numbers are designated as follows: ① malto-dextrin series; ② acceptor product of isomaltulose (ⓐ,ⓐ′,ⓐ′′)*; ③ acceptor reaction product of leucrose (ⓑ,ⓑ′)**; ④ highly branched oligosaccharides; ⑤ isomaltodextrin series; and lane 1-20: acceptor reaction product of dextransucrase and fractions separated from highly branched oligosaccharides.

In the picture, the acceptor products of isomaltulose (ⓐ,ⓐ′,ⓐ′′) are identified with fractions separated from highly branched oligosaccharides and acceptor reaction products thereof. The acceptor products of leucrose (ⓑ,ⓑ′) are identified with fractions separated from highly branched oligo-saccharides and acceptor reaction products thereof.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Example 1. Manufacture of crude enzyme solution

E.coli DH5α/pFMCM(KCTC 0859BP) was inoculated in LB broth(1% trypton, 0.5% yeast extract, 0.5% NaCl) including ampicillin(50μg/ml), and then cultivated at a temperature of 37°C. After only cells were removed from the culture, crude enzyme solution was prepared and then used as dextransucrase.

L. mesenteroides NRRL B-742 was inoculated in LB medium consisting of yeast extract of 5g, peptone of 5g, K_2HPO_4 of 20g, sucrose of 20g in 1L water and then cultivated at a temperature of 37°C. After separation of the cells from the culture, crude dextransucrase solution was prepared and then used.

L. mesenteroides NRRL B-1299 was inoculated in LB medium consisting of yeast extract of 5g, peptone of 5g, K_2HPO_4 of 20g, sucrose of 20g in 1L water, and then cultivated at a temperature of 37°C. After cells were removed from the

culture, crude dextransucrase solution was prepared and then used.

Example 2. Production of oligosaccharides and the identification of reaction product thereof

In order to produce oligosaccharides, 4.5M sucrose solution was mixed with enzyme obtained from Example 1. The final sucrose concentration of enzyme reactor was 0.5~4.0M, and it was made to react at a temperature 28°C.

The enzyme of 0.1~10U/ml was used. The one unit of enzyme activity is represented by the number of μmol of fructose released from sucrose per 1 minute and per 1 ml of enzyme. The reaction was performed until sucrose of the reactor was consumed. After reaction liquid of 1μl was spotted on Merck K6F TLC plate, then the plate was developed twice in MeNO/1-propanol/water (2/5/2.5, v/v/v). Here, the component of carbohydrate separated from the TLC plate was identified by using dipping reagent comprising α-naphthol of 0.5%(w/v) and sulfuric acid of 5%(v/v). In this way, the production of oligosaccharides and the use of sucrose were identified. Each quantity of carbohydrate was analyzed by NIH Image Program in McIntosh (Power PC; 7100/80) computer. The structure of the product was identified by HPLC.

E.coli DH5α/pFMCM dextransucrase was used as enzyme solution, and it was made to react under the above synthesizing condition. Then, the carbohydrate components and the distribution of the synthesized products were identified by TLC and HPLC. The result is shown in Figures 1 and 2.

L. mesenteroides NRRL B-742 dextransucrase was used as enzyme, and sucrose solution of 4M, 2M and 1M was used as substrate. After they were made to react under the above condition, the carbohydrate components and the distribution

of the synthesized products were identified by TLC. The result is shown in Figures 1 and 3.

L. mesenteroides NRRL B-1299 dextransucrase was used as enzyme. Reaction enzyme unit was one and the final sucrose concentration was 0.1~3.09 M. After it was made to react under the above condition, the carbohydrate components and the distribution of the synthesized products were identified by TLC. Maltose was added with 1/10 and 1/100 of the sucrose concentration. The result is shown in Figure 4.

Example 3. Identification of thermostable and acid-stable oligosaccharides

E.coli DH5α/pFMCM dextransucrase of 120U(1ml) obtained from Example 1 was added to 4M sucrose of 1000 ml, and it was made to react at a temperature of 28°C. As a result, enzyme reaction solution was obtained.

Each enzyme reaction liquid of 50ml was prepared into solution of pH 2, 3, 4, or 5. Each set was divided into 5 and incubated at a temperature of 40, 60, 80, 100 and 120°C for 15 minutes, and then quenched. The pattern of oligosaccharides in each enzyme reaction solution was hardly changed as shown in the TLC result. The result is shown in Figure 5.

The enzyme reaction solution of 50ml was set at a temperature of 120°C, 140°C and 160°C, respectively, for 30 minutes and for 1 hour. Then, it was quenched. The pattern of oligosaccharides in enzyme reaction solution was hardly changed as shown in the TLC result. The result is shown in Figure 6.

Example 4. Isolation of oligosaccharides and analysis of its structure

1) Oligosaccharides synthesized by dextransucrases and

sucrose having high concentration were divided into different kinds of saccharides. The structure was analyzed with various ways in order to find out the component of oligosaccharides.

2) Experimental method

a. Isolation of oligosaccharides using thin layer chromatography

After oligosaccharides were diluted the diluted solution of 1μl was spotted on preparative silica gel plates. It was developed using developing solvent (acetonitrile/ethylacetate/1-propanol/water = 85/20/50/50), and then its lateral side was cut off. Here, the separated carbohydrate component was identified by using dipping reagent comprising N-(1-naphthyl) ethylene-diamine of 0.3% (w/v) and sulfuric acid of 5% (v/v). When the plate was developed and then the cut plate was compared with the middle silica gel plate which was not developed. Then, silica gel of each fraction was gathered. Then, it was solved in the same developing solvent. After it was centrifuged, the same developing solvent was applied. The solution was dehydrated and then the dried carbohydrate powder was dissolved in water. Its separation was identified by using the thin layer chromatography.

b. Identification of the structure of oligosaccharides

① The two-dimensional thin layer chromatography: To analyze the structure of oligosaccharides, different developing solvent was used into both directions. Then, the structure of pure shaccharides was identified by separating each saccharide independently. The separated carbohydrate was identified by changing the order of various kinds of developing solvent under the different conditions (b→a, b→c, a→c).

First, oligosaccharides were spotted on TLC plate and

developed in a first developing solvent. After the TLC plates were completely dried, the plate was rotated in 90° and the standard material was spotted on both ends of oligosaccharides. Then the plate was developed in a second developing solvent. The components of carbohydrate separated from the TLC plate were identified by using the dipping reagent comprising N-(1-naphthyl) ethylene-diamine of 0.3% (w/v) and sulfuric acid of 5% (v/v).

Among the above-used developing solvent, a) was acetonitrile/ethylacetate/1-propanol/water=85/20/50/50, b) was nitromethane/1-propanol/water=2/5/1.5, and c) was acetonitrile/ethylacetate/1-propanol/water=85/20/50/70.

② The mass analysis: The degree of polymerization in oligosaccharides was identified by using MALDI-TOF (Finnigan Lasermat 2000 mass spectrometers, CA, USA). After 2, 4-dehydrobenzoic acid(DHB) of 1μl was mixed with 1μl of oligosaccharides which were not separated and with 1μl of each fraction of oligosaccharides, and it was dried at a temperature of 40°C. Then, it was analyzed. Isomalto-oligosaccharides were also analyzed as comparing materials.

③ Enzymes hydrolyzing different linkages(α-, β-, iso-amylase, α-, β-glucosidase, dextranase, α-amyloglucosidase, invertase) were treated in each fraction of the oligosaccharides under each reaction condition. 1μl of reaction solution was spotted on the silica gel plate and then developed twice in nitromethane/1-propanol/water (v/v/v, 2/5/1.5) to identify the separated carbohydrate. The component of carbohydrate separated from the silica gel plate is identified by using the dipping reagent comprising N-(1-naphthyl) ethylene-diamine of 0.3% (w/v) and sulfuric acid of 5% (v/v). The structure was predicted using the result of each hydrolyzing enzyme reaction.

④ Acidic hydrolysis: Each fraction was mixed with 1M hydrochloric acid. After it was hydrolyzed at a temperature of 100°C for 30 minutes and vacuum dried, it was dissolved in water. 1μl of the reaction solution was spotted on the silica gel plate and developed twice in acetonitrile/water (v/v, 85/15). In this way, the structure of mono-saccharides was identified.

⑤ Acceptor reaction: Each fraction was mixed with dextransucrase and 100mM sucrose at the proportion of 1:1:1. It was then made to react at a temperature of 28°C for 12 hours. The product obtained from the above reaction was identified by the thin layer chromatography. After the product was compared with oligosaccharides, each acceptor was identified whether the oligosaccharides are generated by the reaction.

The experimental result is shown in Figures 7-10.

Figure 7 is a picture illustrating the separating result of highly branched oligosaccharides using two-dimensional thin layer chromatography, wherein the reference numbers are designated as follows: lane 17: maltodextrin series; lane 18: highly branched oligo-saccharides; lane 19: the starting point of highly branched oligosaccharides in two-dimensional thin layer chromatography; lane 20: isomaltodextrin series; lane 21: highly branched oligosaccharides using developing solvent as 2:5:1.5 (v/v/v) nitromethane/1-propanol/water(the first development)→85:20:50:50 (v/v/v/v) acetonitrile/ethylacetate/1-propanol/water(the second development); and 1-16: each component material in highly branched oligo-saccharides.

Fractions 1, 4, 8, 12, 16 and polysaccharides were

identified by comparison with the standard materials (lanes 17, 20).

Figure 8 is a picture illustrating the analysis result using the thin layer chromatography of degradation product after the hydrolysis enzyme treatment of fractions separated from highly branched oligosaccharides, wherein the reference numbers are designated as follows: lane 1: maltodextrin series; land 2, 19: highly branched oligosaccharides; lane 20: isomaltodextrin series; (1) lane 3-10: hydrolysis enzyme reaction product of fraction 2 separated from highly branched oligosaccharides; lane 11-18: hydrolysis enzyme reaction product of fraction 3 separated from highly branched oligosaccharides; (2) lane 3-10: hydrolysis enzyme reaction product of fraction 8 separated from highly branched oligosaccharides; and lane 11-18: hydrolysis enzyme reaction product of fraction 9 separated from highly branched oligosaccharides. Each fraction was treated with each hydrolysis enzyme such as α-, β- and iso-amylase, α- and β-glucosidase, dextranase, α-amyloglucosidase, and invertase.

Fractions 2, 3, 5, 8, 9 and 13 were identified by comparison with the reaction product of hydrolysis enzyme for the above carbohydrate solution.

Figure 9 is a picture illustrating the analysis result using the thin layer chromatography of each fraction that was separated from highly branched oligosaccharides and treated with dextranase, wherein the reference numbers are designated as follows: lane 1: isomaltodextrin series; lane 2: maltodextrin series; lane 3: highly branched oligosaccharides; lane 4: dextranase degradation product of fraction separated from highly branched oligosaccharides; lane 5: degradation product after isomaltulose was treated with dextranase; lane 6: degradation product after leucrose was treated with dextranase; lane 7: highly branched

oligosaccharides; and lane 8: isomalto-oligosaccharides.

Fractions 6, 7, 10, 11, 13, 14 and 15 are identified by comparison with the dextranase hydrolysis products of fructose compounds.

[Table 1] Degradation product after hydrolysis enzyme treatment of products separated from highly branched oligosaccharides.

Enzyme		Components of separated carbohydrate															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
α-amylase		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-amylase		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
iso-amylase		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-glucosidase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β-glucosidase		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
amyloglucosidase		-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
invertase		-	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-
Dextranase	1	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	2	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
(formation of	3	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-
degradation	4	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+
product after	5	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
reaction for	6	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
treating	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
dextra-nase)	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

In the table, "+" means that there is degradation product while "-" means that there is no degradation product.

The above table shows the results of Figures 8 and 9. Here, the hydrolysis pattern of enzymes is identified.

Figure 10 is a picture illustrating the analysis result via the thin layer chromatography of the acceptor reaction product using dextransucrase of fractions separated from highly branched oligosaccharides, wherein the reference numbers are designated as follows: ① malto-dextrin series; ② acceptor reaction product of isomaltulose (ⓐ,ⓐ',ⓐ'')*; ③ acceptor reaction product of leucrose (ⓑ,ⓑ')**; ④ highly

branched oligosaccharides; ⑤ isomaltodextrin series; and lane 1-20: acceptor reaction product of dextransucrase in fractions separated from highly branched oligosaccharides.

In the picture, the acceptor reaction products of isomaltulose (ⓐ,ⓐ′,ⓐ′′) were identified with fractions separated from highly branched oligosaccharides and acceptor reaction products thereof. The acceptor reaction products of leucrose (ⓑ,ⓑ′) were identified with fractions separated from highly branched oligosaccharides and acceptor reaction products thereof.

In Figure 10, the structure of small oligosaccharides can be identified by comparison with the acceptor reaction product in reaction with sucrose using dextransucrase.

The oligosaccharides of the present invention which were synthesized by the above methods are composed of about over 17 components. The below table 2 shows the structure of oligosaccharides by comparison with thin layer chromatography, MALDI-TOF results, analysis of products by hydrolysis enzymes and acidic hydrolysis, and reaction products of acceptors.

[Table 2] Structure and quantity of components in highly branched oligosaccharides

Fraction of oligo-saccharides	Ingredient	Structure of ingredient	Quantity (%)
1	D-fructose	Frc	13.5
2	Isomaltulose (6-α-D-glucopyranosyl-D-fructopyranose)	α-Glc(1→6)Frc	4.6
3	Leucrose (5-α-D-glucopyranosyl-D-fructopyranose)	α-Glc(1→5)Frc	12.4
4	Isomaltose	α-Glc(1→6)Glc	7

5	3^2-O-α-D-glucosyl-isomaltose	α-Glc(1→6)Glc α-Glc(1→3)↗	5.2
6	6-α-isomaltosyl-D-fructose	α-Glc(1→6)α-Glc(1→6)Frc	3.7
7	5-α-isomaltosyl D-fructopyranose	α-Glc(1→6)α-Glc(1→5)Frc	4.3
8	Isomaltotriose	α-Glc(1→6)α-Glc(1→6)Glc	6.3
9	3^2-O-α-D-glucosyl-isomaltotriose	α-Glc(1→6)α-Glc(1→6)Glc α-Glc(1→3)	2.9
10	6-α-isomaltotriosyl-D-fructose	[α-Glc(1→6)]$_2$ α-Glc(1→6)Frc	3.3
11	5-α-isomaltotriosyl-D-fructopyranose	[α-Glc(1→6)]$_2$ α-Glc(1→5)Frc	3.8
12	Isomaltotetraose	[α-Glc(1→6)]$_3$Glc	6.2
13	3^2-O-α-D-glucosyl-isomaltotetraose	[α-Glc(1→6)]$_3$ αGlc(1→6)Glc α-Glc(1→3)↗	2.8
14	6-α-isomaltotetraosyl-D-fructose	[α-Glc(1→6)]$_3$ α-Glc(1→6)Frc	2.7
15	5-α-isomaltotetraosyl-D-fructopyranose	[α-Glc(1→6)]$_3$ α-Glc(1→6)Frc	2.7
16	Isomaltopentaose	[α-Glc(1→6)]$_4$Glc	5.6
17	Oligosaccharides Having DP of over 5		8.4
Poly-saccharides	Dextran	[α-Glc(1→6)]$_n$Glc	4.6

INDUSTRIAL APPLICABILITY

The present invention provides a method for manufacturing oligosaccharides from high concentration of sucrose. Since they are stable in acid and heat treatment, the oligosaccharides are suitable for sweeteners in beverages required to be thermostable and acid-stable.

What is claimed is:

1. A method for manufacturing thermostable and acid-stable oligosaccharides, comprising the steps of cultivating Leuconostoc mesenteroides sp. in 0.5-5M sucrose solution and recovering the produced oligosaccharides.

2. A method for manufacturing thermostable and acid-stable oligosaccharides, comprising the steps of adding dextransucrase derived from Leuconostoc mesenteroides sp. in 0.5-5M sucrose solution and recovering the produced oligosaccharides.

3. The method according to claims 1 or 2, wherein the cultivating condition and the solution further include maltose.

4. Oligosaccharides manufactured by the method according to claims 1 or 2.

FIG. 1

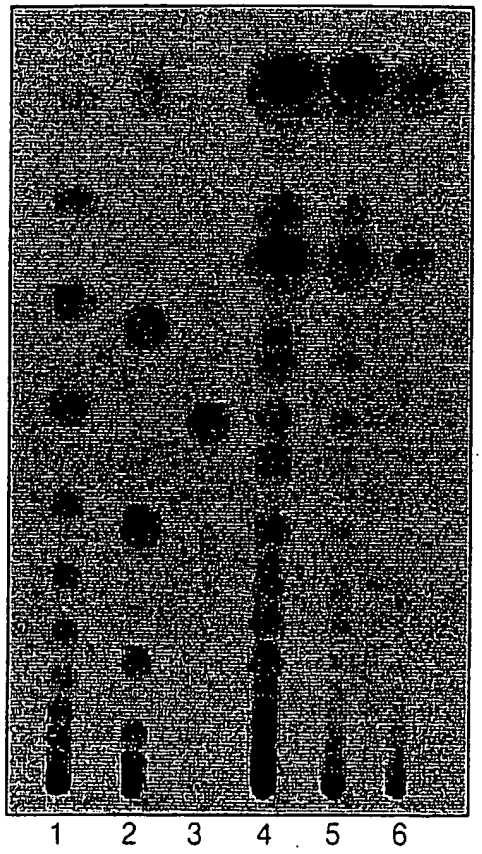


FIG. 2

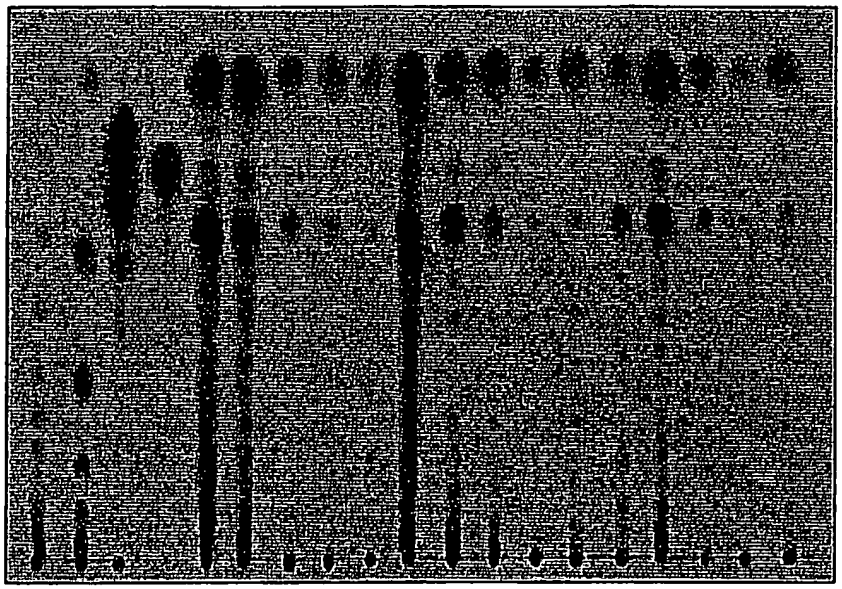


FIG. 3

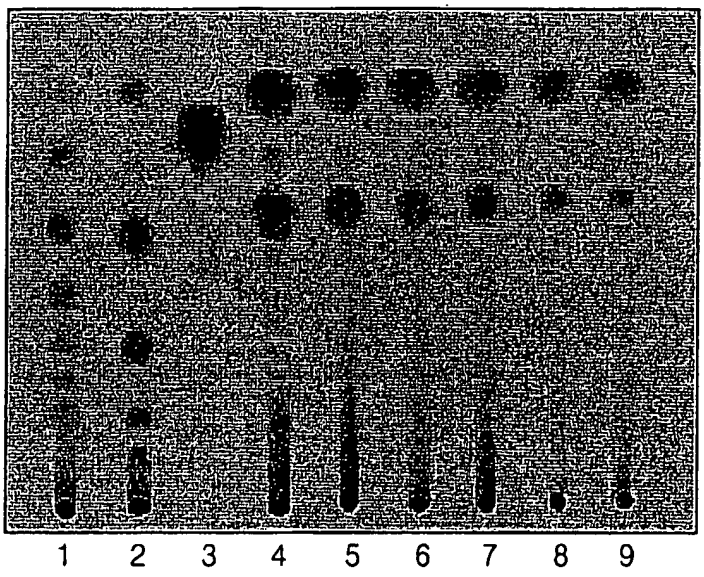


FIG. 4

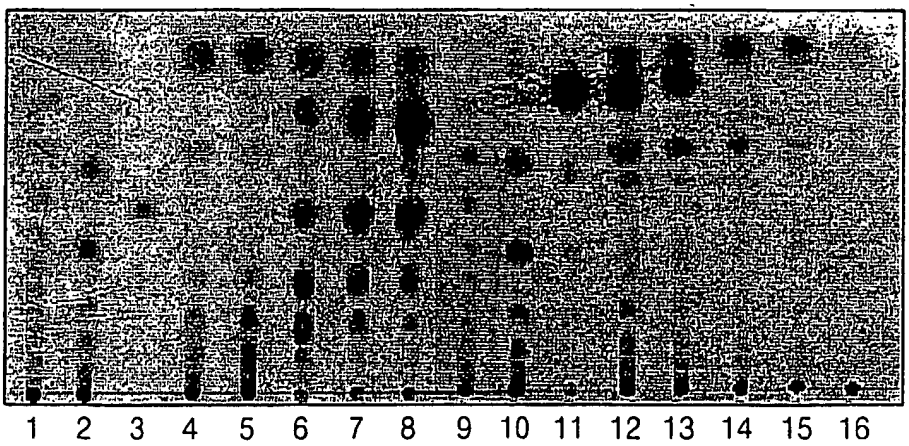


FIG. 5

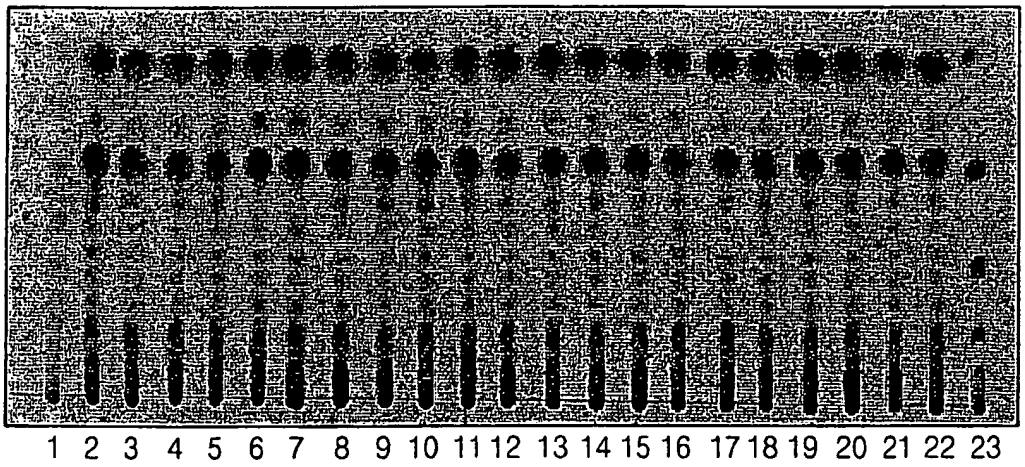


FIG. 6

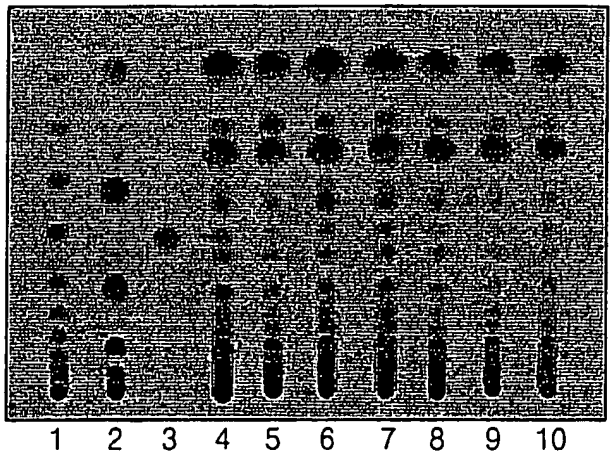


FIG. 7

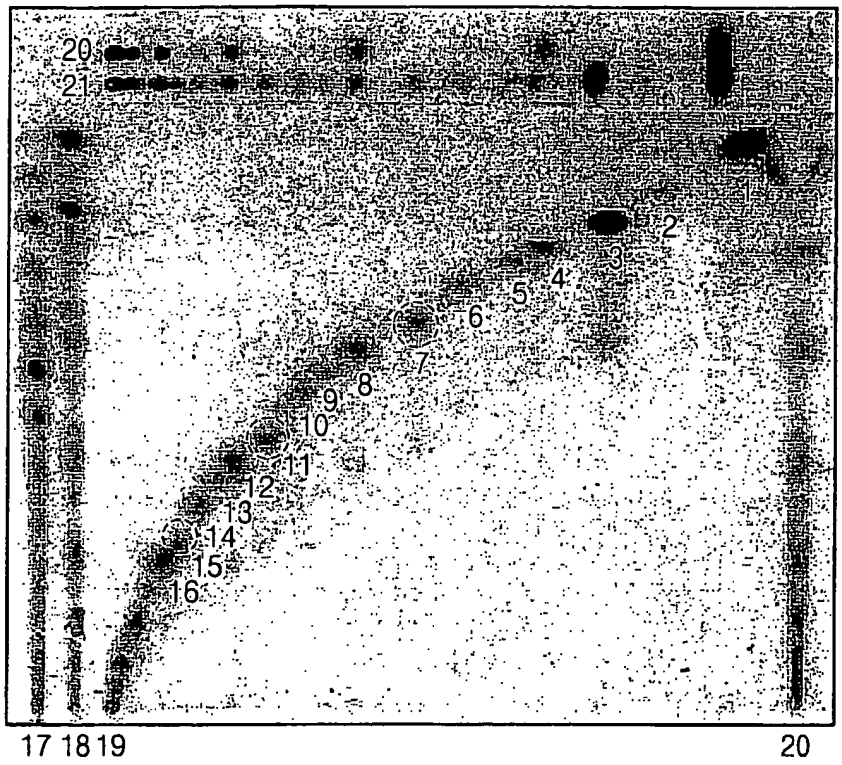
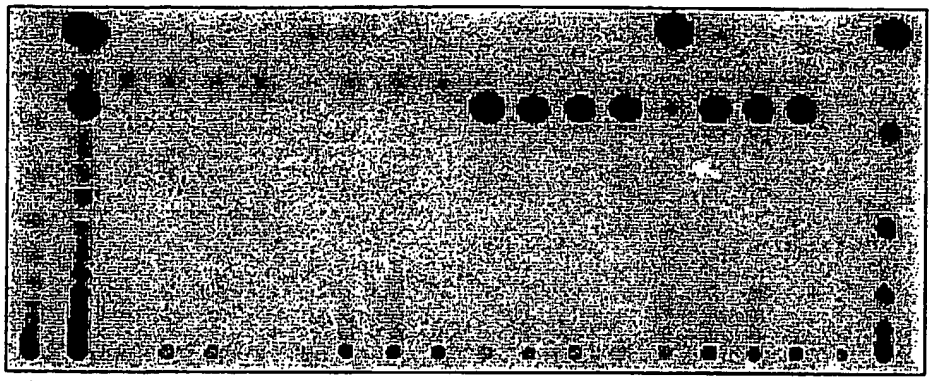


FIG. 8

(1)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

(2)

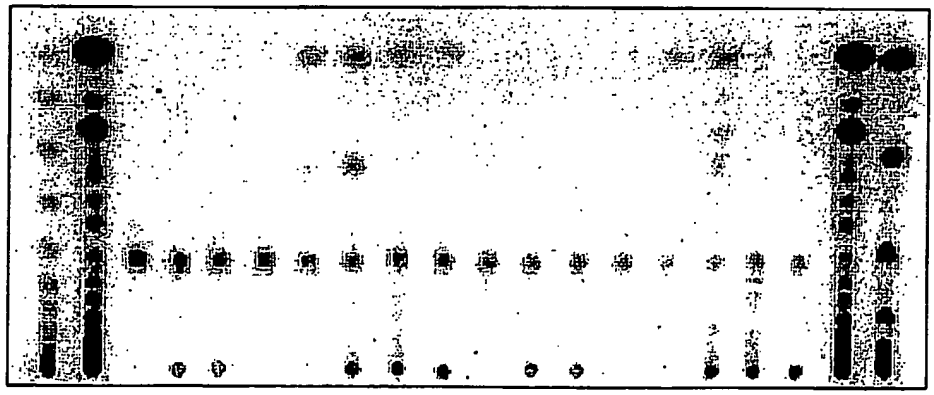

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIG. 9

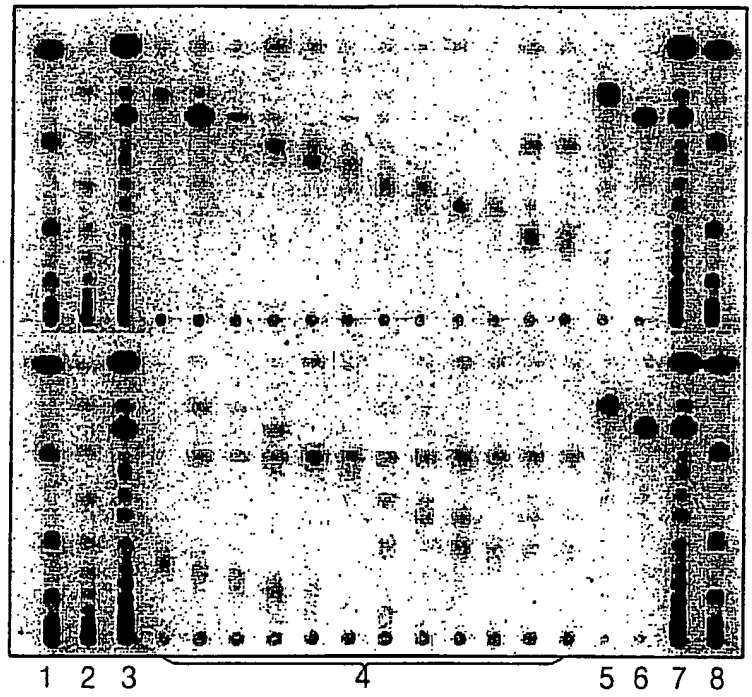
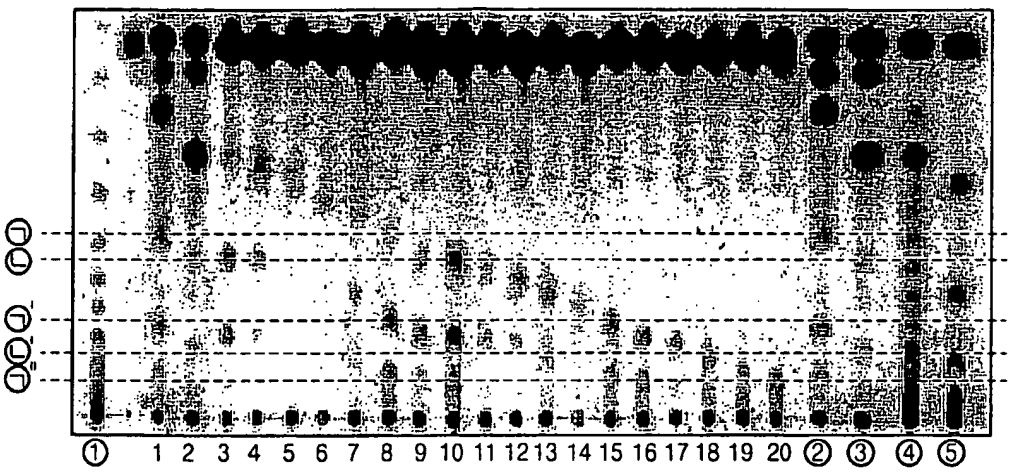


FIG. 10



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR02/00164

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12P 19/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC7 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Patents and applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)
CA, KIPASS, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Heo, Soo Jin et al 'Development of a mixed-culture fermentation process and characterization for new oligosaccharides and dextran using Lipomyces starkeyi and Leuconostoc mesenteroides' Sanop Misaengmul Hakhoechi, 27(4), pages 304-310, 1999 see the abstract	1-4
X	Pereira, A. M. 'In vitro synthesis of oligosaccharides by acceptor reaction of dextransucrase from Leuconostoc mesenteroides', Biotechnol. Lett., 20(4), pages 397-401, 1998 see the pages 397-398	1-2, 4
Y	Heinche, Kristin 'Kinetics of the dextransucrase acceptor reaction with maltose-experimental results and modeling', Enzyme. Microb. Technol., 24(8/9), pages 523-534, 1999 see the pages 526-527	1-4
Y	Seo, Hyun Chang, 'Structural characteristics of novel branched oligosaccharides synthesized by a maltose acceptor reaction with dextransucrase from Leuconostoc mesenteroides M-12' Korean J. Food & Nutr. Vol. 10, No. 1, pages 102-109, 1997 see the whole document	1-4

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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